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Investigation of Biofilm Formation and Control for Spacecraft –An Early Literature Review

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Bacterial biofilms are an important and often problematic aspect of life on earth and in space. Biofilms of opportunistic pathogenic bacteria can lead to severe and costly contamination problems that directly affect human health and long-term mission planning. Microbial contamination on board the International Space Station (ISS) continues to pose mission risks, both to crew health and hardware reliability. In order to optimize the design of future space exploration vehicles, a thorough understanding of biofilm formation and control technologies is needed to control the habitat's microbial environment. This paper provides a literature review on microbial behavior, biofilm formation in spacecraft or simulated spacecraft environments, and the state of the art of biofilm prevention mechanisms.

Nomenclature

AES	=	Advanced Exploration Systems
AIAA	=	American Institute of Aeronautics and Astronautics
AR	=	acid resistance system
AR2	=	acid resistance system 2
ATCC	=	American Type Culture Collection
cDNA	=	complementary deoxyribonucleic acid
CFU	=	colony-forming unit
CWC	=	contingency water containers
DNA	=	deoxyribonucleic acid
EFA	=	External Filter Assembly
EMU	=	Extravehicular Mobility Unit
EPS	=	extracellular polymeric matrix
FPA	=	fluid processing apparatus
g	=	force of Earth's gravity
GE	=	gas exchange
HARV	=	high aspect ratio vessel
ISS	=	International Space Station
LB	=	Lennox Broth
LD_{50}	=	lethal dose that kills 50% of test samples
M9	=	M9 minimal growth media
mAUM	=	modified artificial urine medium

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MLS = Mostly Liquid Separator
mRNA = messenger ribonucleic acid
NASA = National Aeronautics and Space Administration
OMV = outer membrane vesicle
ORU = Filter Orbital Replacement Unit
PLOS = Public Library of Science
RWV = rotating wall vessel
SEM = Scanning Electron Microscopy
σ^s = Sigma Factor
SLIPS = Slippery Liquid-Infused Porous Surface
SPCU HX = Heat Exchanger
SRV-K = System for Regeneration of Condensate Water
STS = Space Transportation System
SVO-ZV = Russian system for storage and dispensing of ground-supplied water
TOCA = Total Organic Carbon Analyzer
UPA = Urine Process Assembly
UV = Ultraviolet
wca/wza = colonic acid synthesis operon
WPA = Water Process Assembly
WT = Waste Tank

I. Introduction

BACTERIA are nothing short of remarkable in their ability to acclimate to new challenges and thrive in areas previously thought inhospitable to any life form. Found to successfully grow in diverse environments such as the hypersaline hot springs of Mono Lake in California[1] and the acidic Rio Tinto in Spain[2] microbial survival techniques have evolved with them. Their presence has also been noted under microgravity,[3,4] but understanding of their growth and consequent biofilm formation in space are not well understood. Microbial studies of spacecraft such as Salyut 6, Mir, and the International Space Station (ISS) have all shown that biofilms are becoming a higher risk for longer missions. Piping, electrical equipment, hardware, and water systems have all been documented to have microbial growth and biofilm formation, and therefore material damage.[5,6,7,8,9] Furthermore, research into the influence of the space environment on astronaut immune systems has shown detrimental effects which leave them at increased risk of health issues.[10,11,12] Medical issues believed to have been due to microbial influence include urinary tract infections, rashes, allergies, and upper respiratory infections.[13]

In order to develop advanced methods that address the microbial control issue in space, better understanding of microbial behavior in space conditions would be very valuable. Though studies have been done on biofilm establishment in microgravity conditions and on varying spacecraft surfaces, the effects of the space environment on bacteria are yet to be elucidated.[14] In this review we aim to better understand microbial physiology, behavior, and environmental influence in space to help identify potential areas of biofilm mitigation. Subsequently, the different mechanisms which are being utilized to develop state of the art biofilm prevention, control, and destruction technology will also be briefly overviewed.

II. Bacterial Contamination in Spacecraft

Microbial growth in space has been documented across missions and spacecraft for years in an attempt to mitigate contamination. Biofilms could lead to problems ranging from material damage to medical issues in longer missions in which the same methods of regulation currently used cannot be upheld because of resupply limitations. When coupled with the trend in research revealing detriments in astronaut immune systems, the issue could be compounded during extended space travel. Currently the effects noted have been more damaging to hardware than astronauts. For instance, Weir et al.[9] discovered that biofilms had been the source of Water Process Assembly (WPA) issues. There had been changes in water pressure between the Waste Tank (WT) and Mostly Liquid Separator (MLS) in 2010 which led to having to compensate with changes in water flow in other areas to compensate. Once removed, it was shown that the MLS inlet solenoid valve was clogged by fungi and bacteria biofilms. Addition of a filter between the WT and MLS in the form of an External Filter Assembly (EFA) allowed for less clogging of the MLS. A year after installation problems with water pressure once again indicated changes in the system. Swapping of the filter for a new

one and consequent study of the old one led to the conclusion that once again biomass had accumulated and created pressure change issues (Figure 1).

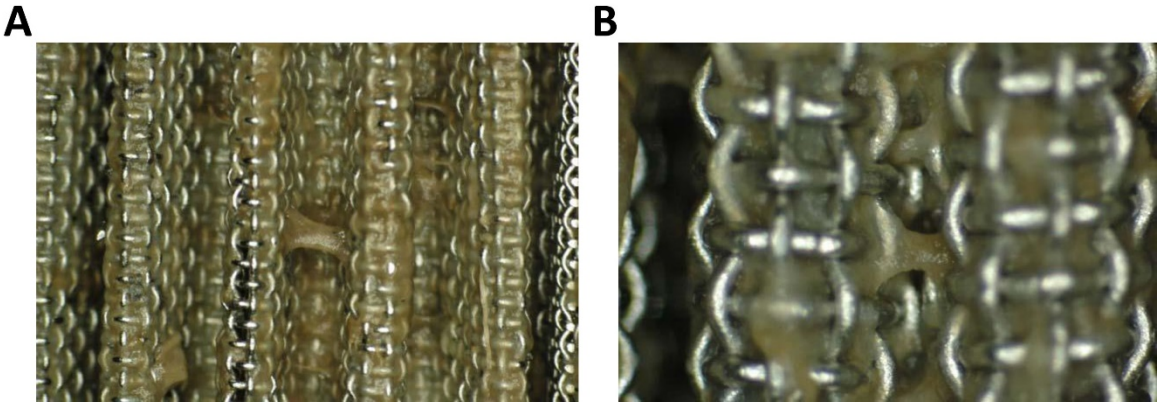

Figure 1. Biofilm formation on the EFA filter inlet mesh at A) ~35x and B) ~50x magnification.[9] These figures are taken from [Microbiological Characterization of the International Space Station Water Process Assembly External Filter Assembly S/N 01, N. Weir, M. Wilson, A. Yoetz, T. Molina, R. Bruce, G. Sitler, L.Carter] and used with permission of NASA and AIAA.

A biofilm is the clustering of planktonic microbes in a sessile suspension of extracellular polymeric substance (EPS) matrix attached permanently to a substrate surface through interactions with cell surface biopolymers.[15] The jelly-like EPS supports biofilm formation with the aid of microbial secreted factors to coordinate communication, spread nutrients, and buffer microbes from environmental stressors.[16,17] These synergistic characteristics lead to its hardiness and adaptability, leading to health hazards and biofouling of materials critical to long term mission sustainability.[7] And though microbial presence has been noted across air and surface samples as well, this work focuses on the effects of microbes, specifically on water systems, in consideration of their repeated presence in past missions.[18],[19] For instance, the space shuttle *Discovery* potable water samples, collected from the galley during STS-70 in 1995, contained *Burkholderia cepacia* and *Burkholderia pickettii* at a count of about 20 colony forming units (CFU)/100mL. At the time, standards for microbial quality were set with an allowance of less than 1 CFU/100mL of heterotrophic bacteria, less than 1 CFU/100mL of coliform bacteria, less than 1 CFU/100mL of yeasts or molds, and less than 1 CFU/100mL of anaerobic bacteria. Furthermore, across 24 space shuttle missions prior to the ISS, Koenig and Pierson[20] reported that the presence of *B. cepacia* was a common isolate from the main potable water tank (Tank A) which relied on water transported from the ground as the main source. On the space shuttle *Columbia*, bacterial biofilms of the genus *Bacillus* were found in water and waste lines, even though the materials had gone through proper iodine flush treatments.[20] Post-mission testing revealed spore formations which protected the bacteria from the iodine well enough to help them become viable again. Other research by Castro et al.[21] showed how, during missions NASA-6 and NASA-7 on *Mir*, coliform bacteria presence was revealed in condensate which had been pooling due to problems with the environmental control systems.[19] Since condensate is recycled as part of the potable water system, the chance of it reintroducing bacteria into the lines became a potential source of contamination which did not lead to major issues. Most of the bacteria isolated from spacecraft water systems are known to be common strains found in wastewater or soil environments.[22]

When the ISS was initially set up, testing of flight-ready potable water that was ground supplied and shuttle-provided was known to have exceeded bacterial count limitations on several occasions. In these samples, 27 bacteria colony types were present and predominantly Gram-negative. Among those samples, 25% had *Sphingomonas* and 18% showed the presence of *Methylobacterium*,[23] both of which have the potential to be infectious in immunocompromised individuals. Some of the other species such as *Blastobacter denitrificans*, though usually not considered problematic due to their presence in the natural environment, have shown rare occasions of opportunistic infection[24]. Nevertheless, even with these species not being of immediate threat, they do give warning as to the potential of bacterial presence in areas which are not easily open to disinfection, especially if longer term missions are to be considered. Table 1 from the same study gives a more detailed summary of the type of bacteria isolated as well as their locations and sources. This information highlights the importance of diligence in control methods as well as the hardiness of bacteria. Studies in the *Mir* space station have shown the presence of 108 bacterial species in the fifteen year span in which the review by Novikova[25] was based, which included microbial presence in 949 of the 1150

total surface and air samples. And even with constant monitoring and procedures for when contamination rises above acceptable levels, surfaces which seem to be predisposed to microbial growth require replacement or coatings to be more antimicrobial. Tests in the ISS have shown repeated contamination of areas such as a ventilation screen panel and a table surface in the Service Module, as well as behind Functional Cargo Block panels.[26] This could indicate that either the strains on such surfaces have changed enough to succesfully survive or were genetically different in some way prior to innoculation on the prone surface. They could also have higher survivability due to the features in some areas which allow them more surface area (tanks or pipes), ridged surfaces (tank bellows), or nooks to avoid biocides in. It has been hypothesized that bacterial biofilms could acquire developmental changes when introduced to new environmental factors in space such as microgravity, a closed ecosystem, and elevated radiation. However, studies are still being conducted and the extent of influence of space conditions has yet to be fully elucidated.

Table 1. Summary of bacterial isolates found in water samples of various tested potable water hardware.[23] Reprinted by permission from Springer Nature.

Source	*Tracking number*	*Sample origin*	*Identifications*
Humidity condensate processor	W-1	Processed during flight	*Sphingomonas paucimobilis*
SRV-K cold	W-2	Processed during flight	*Sphingomonas paucimobilis*
SRV-K hot	W-3	Processed during flight	*Sphingomonas stygialis*
Filter	W-4	Processed during flight	Unidentified Gram-negative rod
	W-5	Processed during flight	*Bradyrhizobium japonicum* or *Blastobacter denitrificans*
	W-6	Archive	*Sphingomonas paucimobilis*
	W-7	Archive	*Ralstonia eutropha*
	W-8	Archive	*Ralstonia eutropha*
	W-9	Archive	*Sphingomonas stygialis*
	W-10	Collected at Filter Reactor within system	*Ralstonia eutropha*
Water dispensing unit SVO-ZV	W-11	Processed during flight	*Methylobacterium fujisawaense*
	W-12	Processed during flight	*Ralstonia eutropha*
	W-13	Processed during flight	*Bradyrhizobium japonicum*
	W-14	Processed during flight	*Sphingomonas* species
	W-15	Archive	*Methylobacterium fujisawaense*
	W-16	Archive	*Bradyrhizobium japonicum* or *Blastobacter denitrificans*
	W-17	Archive	Unidentified Gram-negative rod
	W-18	Archive	*Bradyrhizobium japonicum* or *Blastobacter denitrificans*
	W-19	Archive	*Methylobacterium fujisawaense*
	W-20	Archive	*Pseudomonas stygialis*
Contingency water containers (CWCs)	W-21	S/N 5110	*Methylobacterium fujisawaense*
	W-22	S/N 5031	*Acinetobacter calcoaceticus or baumannii*
	W-23	S/N 5031	Unidentified Gram-negative rod
	W-24	S/N 5056	*Sphingomonas paucimobilis*
	W-25	S/N 5055	*Microbacterium liquefaciens*, *luteolum*, or *oxydans*
	W-26	S/N 5055*	*Enterobacter* species or *Klebsiella* species
	W-27	S/N 5055*	*Delftia acidovorans*

*Not collected under sterile conditions.

Without formation of biofilms, microbes can still do damage through other means such as byproducts which can lead to corrosion of metals through depassivation of surfaces and production of organic acids.[27] Some persistent bacteria in flight-potable water systems have also been shown to have antibiotic resistance as well as virulence regardless of having levels that remained within safe limits[28], and leading to no directly cited illness in mission crews. However, differentiation between effects seen on planktonic microbes and downstream biofilm formation have not been fully brought to light. There has not been a consensus as to whether adaptations are moderated by change in microbial immediate environment, such as changes in culture medium or nutritional deficiency (indirect influence) much like adaptation responses seen in microbes on earth, or if the changes directly correlate to the change in actual overall environment.[29] Still, certain conclusions have been drawn from past and present research which could help guide future experimentation. For example, most isolated strains from past missions have lineages ubiquitous with human microflora or strains seen in specific clean rooms, proving that the system is indeed a closed enough loop most likely impacted by human presence.[30,31] Furthermore, some studies hypothesize that adaptations of microbial biofilms to their new environment could be comparable to their formation on earth in terms of their physiological reactions to

change. Still, the in-depth details of such mechanisms and their comparison to in-flight growth require further study and cannot therefore be considered analogous as of yet.

III. Bacterial Growth Studies in Microgravity

A. Cell Motility and Reduced Extracellular Transport

Some studies hypothesize that the ability of microgravity to affect biofilm formation could be influenced by the complex interplay of individual microbe motility, the inoculating medium's convection, and the state of the immediate nutritional environment. Benoit and Klaus[32] did a review in which they looked back at previous research of microgravity effects on microbial growth, finding inconsistencies even in studies done with the same microbial strains, which seem to correlate to whether the microbes in question lacked motility. Their review proposes that a trend can be seen in which the availability of nutrition and the capacity to move to such areas becomes key. They state that gravity-induced convection would affect microbes when in a more viscous and denser substrate in ground-based experiments, though this would be a more prevalent effect in non-motile species. In terrestrial conditions, the pull of gravity as well as resistance from the suspension culture would impose low shear forces to non-motile sinking cells while simultaneously supporting the movement of waste and nutrients away with help from diffusion (Figure 1).[33] In this regard, the lack of gravity (and therefore convection) in space would indicate that *non-motile* bacteria experience no sedimentation and no potentially damaging shear force because they would not sink. As an interesting note, Klaus et al.[33] mention the implication that at 1g (Figure 2), with *g* being the force of Earth's gravity, the microbes would be considered "normal," implying that microbes in space in a planktonic state would be "deformed". Though how motility influences deformation of a cell prior to microgravity conditions, as well as how it could be affected in space, has not been an area of focus seen in this research. Furthermore, how this affects the efficacy of the biofilm structure either through biological mechanism changes (gene activation, secretion factors variation) or physical mechanisms (initial microbial attachment strength, overall biofilm structure or mechanics) requires further study in order to optimize biofilm mitigation methods which could hone in and exploit the microbe's mechanical changes. Specifically, biocides which could target the attachment system of bacteria.

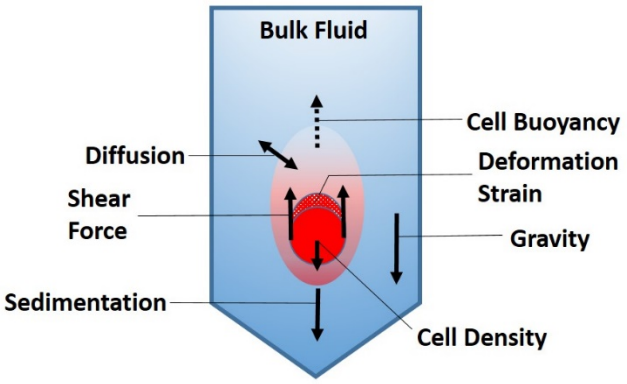

Figure 2. Schematic representation of forces affecting a cell in a 1g viscous fluid environment.

Based on the lack of convection, non-motile microbes in theory could remain spread out when inoculated in a suspension culture in space. However there have been studies indicating that bacteria such as *Escherichia coli*[34] and *Salmonella typhimurium*[35] form clumps which could be the initiating process to biofilm formation for enhanced survival. In a study done by Zea et al.,[34] *E. coli* was sent to the ISS in stasis to be cultured for 49 hours to compare against ground samples grown similarly in Medium E minimal growth medium supplemented with 5g/L of glucose and challenged with gentamicin. They found that cell size of anaerobically grown *E. coli* decreased in comparison to ground control samples, with an average of 59% length and 83% diameter size of those on Earth.[34] According to Zea and colleagues,[34] this is perhaps due to the reduced exposure to nutrients, because of lower surface area, and an increased acidic level brought on by stimulated metabolism processes leading to byproduct buildup. Since shear forces are not available in microgravity, the only force helping cells get distanced from their byproducts would be diffusion. The loss of convection therefore would reduce extracellular transport and create a barrier of detrimental waste around the cells. Interestingly, the addition of gentamicin to samples in microgravity led to, not only the clustering of cells, but creation of outer membrane vesicles (OMV) which have been acknowledged as helpful in biofilm formation. *S. typhimurium* showed similar clumping in a study by Wilson et al.[35] which grew the *S. typhimurium* derivative of SL1344 for 25 hours in Lennox Broth (LB) in a fluid processing apparatus (FPA) during the STS-115 mission of the Atlantis space shuttle. Though the main purpose of the study was to help elucidate the role of Ribonucleic acid (RNA)-binding protein Hfq in gene expression and virulence of the microbes, their morphological studies indicated a change in aggregation which could be indicative of extracellular matrix formation. The imaging results of flight samples were corroborated by the change in expression of genes known to be linked to surface alterations, specifically for biofilm

formation. However, the microbes were fixed after some time in both studies and therefore any further biofilm development was not reported on. Why some bacteria tend to show beginning stages of clustering and biofilm formation in microgravity conditions while others could be showing a tendency to rely more on their byproducts has not, to our knowledge, been studied. Understanding this could offer some insight into microbial behavior and how to reliably determine and compare which microbes would be more likely to turn to such mechanisms of survival, after how long, and how best to control them.

Studies into microbial growth phases of various non-motile bacteria have shown decreased lag phase, extended log phase, and an increased final cell count in comparison to ground controls, which some researchers believe tie into the effects of convection loss and extracellular fluid changes.[31,32] Though this sets a precedent as to the effects of microbial waste and environment on survival, research into how byproduct dispersal and nutrient changes affect other species of bacteria in space remains to be explored more in-depth, especially in an environment such as spacecraft water pipes and tanks, where the level of flow could impact possible growth mechanisms. The effects of convection loss seem not to be as apparent in motile bacteria, which had no major alteration from their ground sample counterparts in various studies.[32] Benoit and Klaus[32] state that, since motile bacteria could agitate the environment around them in suspension cultures and travel away from their byproducts, they would not see the same effects as their non-motile counterparts. Research done by Kacena and Todd[36] seems to corroborate such a claim based on their study on the growth of low motility *E. coli* (ATCC 4157) and swarming *B. subtilis* (ATCC 6051) on 2% bacto-agar supplemented with glucose and Medium E solid agar substrates on mission STS-63, ground controls, and clinorotation. Samples on STS-63 showed a shortened lag phase but unchanged log phase or final cell growth rate when compared to their ground counterparts. This could have been due to the inability of either strain to move fully in their environment, which ultimately restricted the amount of nutrients available to the bacterial colonies in a similar way cultures are restricted on earth. Another study from Kacena et al.[27] utilized a solid agar inoculation of the same motile species of *B. subtilis* (ATCC 6051) and *E. coli* (ATCC 4157) aboard several missions (STS-60, STS-62, STS-63, and STS-69) on Medium E minimal growth medium in FPAs. Under these same conditions, they also ran ground samples under influence of slow rotation, agitation, and static movement. Their findings indicated that final cell population differences between ground samples and those in space conditions had no major variance in growth rate and cell concentration for *B. subtilis* and *E. coli*.[27] These studies therefore suggest that, even though motility plays a role in changing growth, the immediate environment could influence change as well. Clarification as to how high of an impact these play into the development of the subsequent biofilm will be required before further conclusions can be drawn. Conversely, why inertial forces did not seem to have detectable impact on growth require further study.

Brown and colleagues[37] tried to focus on influence from environmental changes to explain why work done by others has seen increases of bacterial growth, even with the possible boundary created by cell waste. Their research with low-motility *E. coli* cells (strain ATCC 4157) compared flight samples with those on Earth after 9 days of growth.[37] Having been suspended in minimal growth medium with glucose supplementation, both sets of samples consumed the glucose completely within the nine days. Therefore, the differences in growth could not be attributed to any difference in the amount of glucose provided—making the growth noted in flight samples more likely to be from gaining higher metabolic efficiency. To further study the effect, they subjected other ground samples to 50g in a centrifuge for separation of the byproducts through sedimentation. Their results indicated that, in comparison to samples grown in 1g, cell populations and efficiency of their growth was decreased. Brown and colleagues[37] state that some of the microbial byproducts have been shown to benefit bacterial growth and therefore could explain the increase seen in microgravity conditions for non-motile species. Still, to our knowledge, the qualitative determination of the effect of byproduct influence in the immediate environment on microbial and even biofilm formation has yet to be determined. Kim et al.[38] further elucidated that the nutritional value of the medium is important in determining microbial behavior in space as well. By controlling the phosphate concentration, carbon source, and oxygen availability, while documenting the motility of *Pseudomonas aeruginosa*, their work indicated that utilization of low oxygen and phosphate led to a decrease in the effects on cell density when in modified artificial urine medium in space (mAUM).[38] The implication that can be drawn from the result is that the limitation of nutrient availability could cause bacteria to become more efficient with their sources, therefore inducing the changes reported in other studies. Variations in the carbon source with ground experiments have been shown to cause changes in *P. aeruginosa* growth.[39,40] However, in their samples, Kim et al.[38] found that, regardless of the change in carbon source, the most important distinction made was that lower oxygen and phosphate levels led to better growth density of the planktonic cells, while increasing either level led to growth more synonymous with ground samples. Though this is only one study, others have shown similar results in the sense that changing nutrient availability altered microbial behavior.[32,41] And though it is difficult to draw conclusions for all the microbes with documented presence in spacecraft, looking further into how nutrient availability influences growth of both microbes and biofilms could offer an alternative source

of control, such as nutrient deprivation, in systems where other methods of biofilm mitigation have not been as successful.

Another study by Kim et al.[42] found that the biofilm production for motile bacteria rose 3-fold in space conditions aboard Atlantis Space Shuttle missions STS-132 and STS-135, increasing not only the biomass but the mean thickness and viable cell count as well, regardless of phosphate or carbon changes. They utilized the opportunistic pathogen *P. aeruginosa* strain PA14 as three different types: wild-type, *ΔmotABCD* (lacking flagella-driven motility), and *ΔpilB* (deficient in pili-dependent motility) on a mixed cellulose ester membrane disc in mAUM. Use of the disc was to ensure biofilm formation on a surface since biofilms have been documented to grow on equipment in space this way. Planktonic cells showed no difference in growth, implying that the increases seen in biofilm biomass were not all due to bacteria growth input. Interestingly, the formation of a distinct cap-and-column biofilm structure seen in their work seemed to be independent of carbon source but reliant on motility. This was in contrast to biofilms on earth which have been known to depend on glucose as a carbon source for similar mushroom-like biofilm structure formations, such as those in the hydrodynamic environment of flow cells. Therefore, Kim and colleagues[42] proposed that the cap-and-column structure is not fully reliant on either the effect of motility or nutrition but on whether its environment is hydrodynamic or static as well. They suggest that the mushroom-like structure seen on earth is just an inhibited version of the cap-and-column formation seen in microgravity because of shear forces in solution potentially limiting mushroom cap growth in between columns (Figure 3). However, the differences seen between samples in FPAs with gas exchange (GE), which showed more biofilm growth with the GE inserts but no difference between structures in ground and flight samples. Both were reported to be flat and dense. The change to flight samples with GE inserts could then be due to an increase in oxygen availability, though it is deduced that an oxygen gradient might exist, which would indicate that mainly surface bacteria nearest to the inserts would change their behavior.

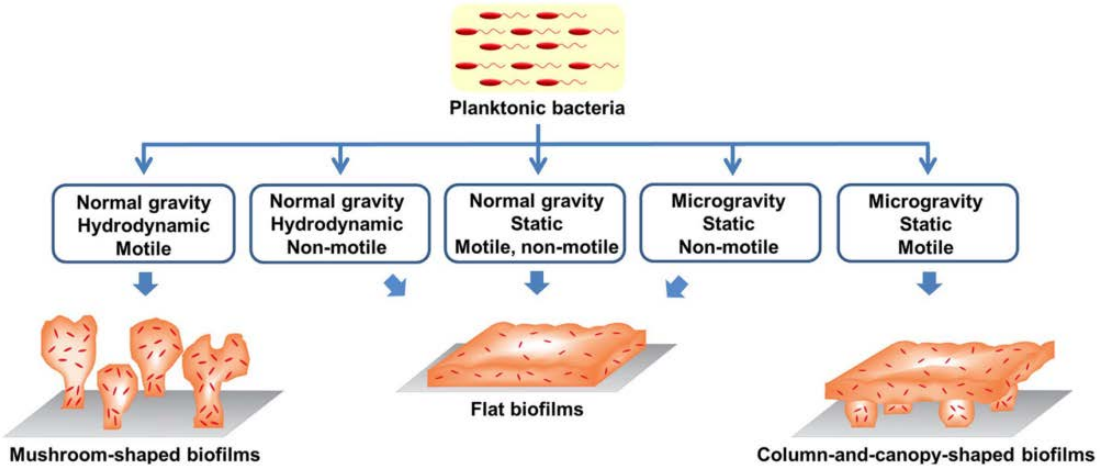

Figure 3. Schematic of potential influences such as gravity, solution flow, and cell motility and how they could alter biofilm development.[42] Reprinted by permission from: Public Library of Science (PLOS).

Shear forces have been previously shown to be influential on *Pseudomonas aeruginosa* even when the carbon source was not a variable to influence biofilm structures. Biofilms were grown by Stoodley et al.,[43] using multiple strains of *P. aeruginosa* in glass flow cells and subjected to either laminar or turbulent flows to study biofilm development under normal 1g conditions. They found that biofilms in turbulent flowing solution had stronger attachment than those with lower shear force applications. Furthermore, when subjected to higher shear for only seconds, biofilms showed a response that was more nonlinear elastic whereas longer time periods induced a viscous flow response. The flow of solution further impacted growth of the biofilms through their structure. In the high shear environment they had more streamer-like structures as opposed to the mound-like shapes seen under low shear. Though biofilm formation has been noted in multiple missions, the study of their physiological aspects as well as the type of nutrition they are taking advantage of to grow in such areas has not been researched together. Trying to take into account more of the environmental details, in which such biofilms have successfully grown could help determine whether the pattern seen in these studies could be tied together or lead to a method of testing such ideas under microgravity conditions that is more standardized.

B. Microbial Genetics, Virulence, and Antibiotic Resistance

Virulence and antibiotic resistance have been shown to increase in space conditions for several bacteria, though whether the effect applies universally, the mechanisms behind such changes, and the pattern of influence on gene expression have been harder to elucidate.[14] With biofilms being communities of varying bacteria, swapping genetic material in the safety of the biofilm environment leads to more resistant planktonic microbes. Establishing the differences between microgravity and ground bacterial growth could help open up a new avenue of bactericidal activity aimed specifically at the mechanisms supporting microbial capacity to activate or swap genes for antibiotic resistance.

Work by Wilson et al.[35] has shown a change of virulence in cultures of motile *Salmonella typhimurium* grown in LB medium during space shuttle mission STS-115. In their study, *S. typhimurium* was used to infect mice orally, with some mice taking increasing doses of either ground or flight cultures for 30 days. The ground samples also had cultures inoculated into FPA and were coordinated for activation and termination of growth in the FPA in-flight. They found that the mice infected with flight samples had increased percent mortality in comparison to those with ground cultures at each infection dosage, shorter time to reach death when using 10^7 dosage, and a value of LD_{50} that was decreased in comparison to ground control mice. Therefore, mice introduced to the flight sample strains dealt with higher virulence than their ground-culture infected counterparts. To get a better idea as to what had changed genetically, total bacterial RNA was isolated from both flight and ground samples. It was then reverse-transcribed to create single-stranded and labeled cDNA to be cohybridized with different *S. typhimurium* genomic DNA and whole-genome microarray slides. The signal created when hybridized could therefore be quantified for analysis seeking statistically significant change of 2-fold or more difference in gene expression. Overall, their study found 167 genes to have altered expression, of which 69 were up-regulated and 98 down-regulated. Among these genes, several connected to biofilm formation were shown to be altered, including up-regulation of *wca/*wza (a colonic acid synthesis operon), *ompA*, and *fimH*. Some genes influencing cell motility were also indicated to be down-regulated, which might suggest the change from planktonic to sessile microbes initiating biofilm formation. Their work also pointed to Hfq, an RNA-binding protein that helps regulate mRNA translation when responding to envelope stress, as a potential influencer in responses to change in the microbe's environment. The gene results revealed change in expression of 64 genes known to be part of the Hfq regulon as well as overall decreased presence of Hfq.

In a study following these results, Wilson et al.[44] showed that, while genes did demonstrate change in *S. typhimurium*, altering the medium's nutritional resources in combination to spaceflight also affected the level of virulence. Their previous work was done on LB but in this study they compared *S. typhimurium* grown on LB and M9 media, as well as supplementing some of the salts from M9 onto LB to see if the difference in those materials influenced microbial response. When comparing their past work, they found that changes they noted when *S. typhimurium* was grown in LB were not seen in M9 cultures. Instead, in M9 minimal media they noted that the bacterial response of the microbes grown in a rotating wall vessel (RWV) bioreactor (mimicking the low fluid shear environment of space) included a shortened lag phase, shorter generation time, and alterations to the acid response when compared to controls. To confirm their results, their new study used LB, M9, and mixed media (LB with M9 salts) for microbial growth aboard STS-115 and STS 123. When comparing the M9 cultured samples against LB, differences in virulence were found. M9 ground samples and flight samples had no major difference in time-to-death curves between them, whereas LB flight samples showed a decrease in time-to death curves in comparison to LB ground controls. The LD_{50} decrease noted in LB flight-grown microbes was also not consistently seen in M9 samples. In LB supplemented with M9 salts, the virulence behavior was similar to that of cultures grown solely in M9, most noted in the lack of change in LD_{50} in flight versus ground samples. They delved into these results further by attempting to distinguish which of the salts from the M9 media were responsible for the influence through variation of salt amounts included in the LB media. Through this they determined that phosphate from NaH_2PO_4 and KH_2PO_4 changed the acid tolerance. Finally, when comparing all the LD_{50} of flight samples only, the increase in relation to LB in flight was much higher than the increases seen in the comparisons done of the ground samples with their LB ground control growth (Table 2). Therefore, the effect of the nutrition available to the microbes could affect behavior enough to downplay virulence seen in microgravity conditions, even if the environment itself might be exacerbating it. Still, their work determined some common genes found to be influenced by the change in environment across the different culture media, some of which were once again found in Hfq protein regulons. The genes found to change seemed to

be involved with motility (flagella), Hyc hydrogenase formation, ABC transporters, structure of ribosomes, utilization of iron, and the function and expression of small regulatory RNA molecules.

Table 2. Flight and control samples of S. typhimurium grown on M9, LB, or LB-M9 media LD_{50} in comparison to LB-only media. Reprinted by permission from: PLOS.[44]

Media	Growth Location	LD_{50} (CFU)	Fold Increase Relative to LB Media - Flight
LB media	Flight	5.81×10^4	1.0
LB-M9 salts media	Flight	7.45×10^5	12.8
M9 media	Flight	3.30×10^6	56.8
Media	**Growth Location**	**LD_{50} (CFU)**	**Fold Increase Relative to LB Media - Ground**
LB Media	Ground	4.02×10^5	1.0
LB-M9 salts media	Ground	5.73×10^5	1.4
M9 media	Ground	2.30×10^6	5.7

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In another study by Zea et al.,[45] they determined that *E. coli* genes associated with starvation, increase of metabolism, enhancement of acetate production, responses to changes in acidity, and alternative energy source searches were overexpressed in samples grown on the AES-1 mission of the *Cygnus* spacecraft. There were 3 main test groups for *E. coli* (ATCC 4157), with each being subjected to either 25, 50, or 75 µg/mL of Gentamicin Sulfate and cultured in Medium E Minimal medium with glucose in an FPA. Overall, 81 genes were found to overexpress across the three sets, though there was no major differences in expression based on concentration of Gentamicin Sulfate utilized, the final cell count of samples in space generally showed a 13-fold increase in comparison to ground controls and expression of genes known to be utilized, in situations where alternative sources of carbon are available (even though the optimal carbon source for such changes was never introduced in the media for these samples). Zea and colleagues[45] claim that in the search to compensate for the loss in nutrients, the microbes seemed to alter genome transcription to widen the types of carbon sources they could use. Overexpression of genes such as *thiFGHS*, *dps*, *crp*, *glnG*, *nac*, and *poxB* indicated that the bacteria were under starvation conditions. Carbon-source hunting could also be hindered by the diffusion-only mass transport conditions around the microbe's environment. Since their medium included glucose, they focused on glucose catabolism genes and found that of the 26 they had data for, 18 were determined to be overexpressed in space conditions, along with 17 of the 23 other genes associated with metabolism in general. Furthermore, the process of cellular amino acid biosynthesis had genes associated to it that were also overexpressed, leading to increased production of amino and organic acids in microgravity conditions for samples in the 50 and 75 µg/mL groups. And though there are three known systems for acid resistance (AR), only one was determined to be utilized and have overexpression of genes in space conditions. AR2, the system to be found in use by the microbes, had overexpression of genes known to control it including *gadA*, *gadBC*, *gadE*, *gadX*, and *gadW*. Therefore, if this system was in play then it was possible that these were in response to buildup of byproducts synthesized by *E. coli* itself. However, when pH of the bulk fluid was tested and compared between ground and flight samples, there was no statistically significant difference. Zea et al.[45] hypothesize that this could be due to the change in acidity being localized to the cells and that the increased acidity could also be raising virulence of these microbes via toxic byproducts. Interestingly, the results reported by Zea et al.[45] mirrored studies done in which nutrients were supplied in a step-wise fashion (when in comparison to cultures where nutrients were introduced all at once).[46] Such microbial behavior could be due to their metabolism mechanisms becoming more efficient under strained conditions. They also cited the study by Wilson et al.[35] on Hfq and indicated that though the overexpression of 11 of the 12 genes match in over or under-expression trends, Hfq itself was not underexpressed in their work. When considering multiple results across different strains, the response of microbes seems to depend on multiple factors environmentally. Though further clarification of the influence of nutrition is required, altering the availability of nutrients could not only lead to a method of biofilm regulation, but of genetic control as well.

Another consideration when studying the genetics behind microbial biofilms is the high likeliness that the biofilm would consist of a mixture of different bacterial strains that have the ability to do genetic exchanges. For instance, *Ralstonia pickettii* and *Cupriavidus metallidurans* have been found in samples of water from the ISS and are known to be contaminants with megaplasmid genetic codes enabling different resistance genes.[47] Mijnendonckx et al.[47] did a study on the two bacterial strains from space-related environments and found genes for tolerance of metals (including

silver) within the tested plasmids. Their work focused on the persistence of these strains in known space environments and their differences in tolerance to the different methods of disinfection against them, including those commonly seen in space-related work such as UV and silver disinfection. And though both strains are known to be common in water sources and of low health risk, their ability to inherit resistance and pass it on could create a pathway for more aggressive strains of bacteria that might survive. Therefore, taking into account such hardiness, with the ability to swap genetic material in the form of plasmids, transposons, and genetic islands in different strains in a biofilm community will become important in long term mission biofilm mitigation. Studies into how to hinder such processes or even introduce genetic code that could be detrimental to bacterial survival could be another area of mitigation on biofilm growth.

The influence of microgravity on the proteins involved in genetic regulation of biofilms could also be of major interest. The general stress response of bacteria for instance, can be triggered when facing something that challenges microbial survival such as starvation, heat shock, or oxidative stress. In doing so, bacteria have been known to show increased levels of sigma factor (σ^s) which ultimately led to transcription of genes necessary to confer cellular resistance. Lynch and colleagues[48] did studies on *E. coli* strains (AMS6, AMS150, and AMS171) grown in a high aspect ratio vessel (HARV) bioreactor which generated an environment of low-shear simulated microgravity. Utilizing glucose-supplemented M9 media within the HARV, they focused on regulation of σ^s at varied stages of microbial growth. Their work showed that during the stationary and exponential phases, the levels of sigma factor varied. When wild type and AMS150 (σ^s-deficient) strains were subjected to the HARV environment with hyperosmosis or low pH, differences were found in comparison of the exponential and stationary growth phase behavior. Overall, simulated microgravity seemed to offer a raise in resistance to stressors in both mutant and wild-type strains, possibly indicating that the way the strains respond to stressors might be along the same pathway. However they concluded that the effect also depended on the phase of growth. During the exponential phase, resistance was independent from sigma factor levels while the stationary phase was dependent on its presence. Lynch et al.[48] further determined through calculation (via σ^s concentration and half-life, as well as copy number of *rpoS* mRNA) that the translation rate of *rpoS* gene as well as the efficiency of the process were both higher in simulated microgravity cells, though the change was markedly higher during stationary phase growth. From these results they concluded that simulated microgravity affected σ^s by influencing the *rpoS* translation rate efficiency and making the sigma protein itself easier to alter (less stable) during the exponential phase. Instability of the protein in the new environment could likely be due to influence on the folding pattern. Also of note, there is the possibility in nature for conditions in which microbial cells encounter low shear stress and are in stationary phase, similar to their simulated microgravity counterparts. The implication that similar environments could increase resistance to antimicrobials becomes more important both for long term mission sustainability as well as mitigation in the current terrestrial environment. Matin et al.[49] stated that experiments in space have shown crystal formation from proteins occurring more readily, reinforcing the possibility that protein folding influenced the study by Lynch et al.[48] Furthermore, how the interplay of genes is affected by the folding (or lack thereof) of proteins and their further effect on biofilm development is still a growing field of study.

IV. Biofilm Prevention, Mitigation, and Destruction Mechanisms

Approaches to biofilm control in spacecraft water systems have relied on methodology mainly meant to remove already present biofilms.[50] Still, the ISS does rely on detection methods for microbes that include the microbial check valve in the WPA and water sampling with the Total Organic Carbon Analyzer (TOCA), which detects total organic and inorganic carbon available.[51] Detected carbon would imply that there are sources of food present for bacteria and therefore are indicators of potential microbial growth, but are limited in their ability to detect bacteria by their potential food source only. The microbial check valve however can be used for direct detection via flowing of water through a plated media which then requires 48 hours for growth before determination of microbial CFUs. Similarly, swabbing of areas can be done followed by traditional culturing methods. But culturing of bacteria is lacking both due to the need for time, difficulty of access to some locations, and the inability of some bacteria to successfully be cultured. Furthermore, in-depth study of samples can be limited in orbit by lack of equipment.[22] Otherwise, indications of biofilm growth are limited to system effects that then require physical removal of the part for inspection. Though there are surely more methods and equipment of detection being established, they are outside the scope of this paper and therefore not mentioned here.

The idea that biofilm removal can be permanent in a system is not possible, considering the human microflora and equipment handling being constant vectors for potential reintroduction into the system. Though astronauts have been known in past missions to be quarantined for a period of time prior to their missions, isolating them from environmental bacteria is not feasible without a high level quarantine from beginning to end, including not only the people but equipment as well. Iodine is the current water disinfectant in potable water systems on the American side

of the ISS and is effective in keeping bacterial growth to safe levels after going through multiple treatment systems. It also requires use of an internal Filter Orbital Replacement Unit (ORU), which supports filtration by catching particulates and bacteria but must be replaced periodically. As a backup, a mode exists for the system in which the system dispenses a much higher level of iodine to flush in case of system failure to meet criteria. However, even though it is removed prior to use by astronauts, it alters the taste of water and, according to our search, does show some declining biocidal ability.[50,52] The Russian segment of the ISS utilizes a different method, reliant on silver biocide and mineral addition post treatment, with toxicity levels of silver low enough that their consumption by astronauts is deemed safe. However, studies have shown that silver chemical states change, when in contact with various surface materials utilized in water systems, leading to alteration of the surfaces enough that it takes away from silver's biocidal activity.[53]

Finding a method of biofilm prevention or mitigation therefore is critical due to the anticipated extension in mission length, limited detection abilities, and known detriment of the astronaut immune system in space. It would help cut down on mission costs and weight requirements as well as on replacement parts which need to be kept due to the continuous growth of biofilm in the same areas.[14] If missions are to remain sustainable in self-sufficient ways, staying ahead of microbial adaptations is key in keeping all water systems working properly. At the very least, biofilms should be controlled to not outgrow their initial area or slough off into the passing water. When considering factors that could affect the method of prevention and mitigation, the state of the water caused by application of a method (cloudiness, flow rate, temperature, color) and handling materials (geometry and composition) are important. Schultz et al.[6] state that bacteria which are attached to materials have a higher biocidal resistance than planktonic bacteria, a major consideration when trying to figure out a mechanism of control.[6] Toxicity in humans however is one of the foremost limiting factors of biofilm control methods, which in combination with microbial adaptation and resistance, makes it difficult if only one method of biofilm prevention is applied indefinitely. Other considerations of note would include practicality, compatibility, scalability, efficacy, and removal of dead bacteria post-treatment which could act as food sources for the bacteria which follow. Current applications fall under several categories which include physical, chemical, radiation, or biological approaches (Figure 4). A review by Pugel and colleagues[54] outlined many of the current NASA-certified approaches for microbial control on various surfaces in space. Our work will also consider these based on their mechanisms in the water system, as well as other studies which are seeing further development either for longer term missions in space or which show potential past application research done terrestrially.

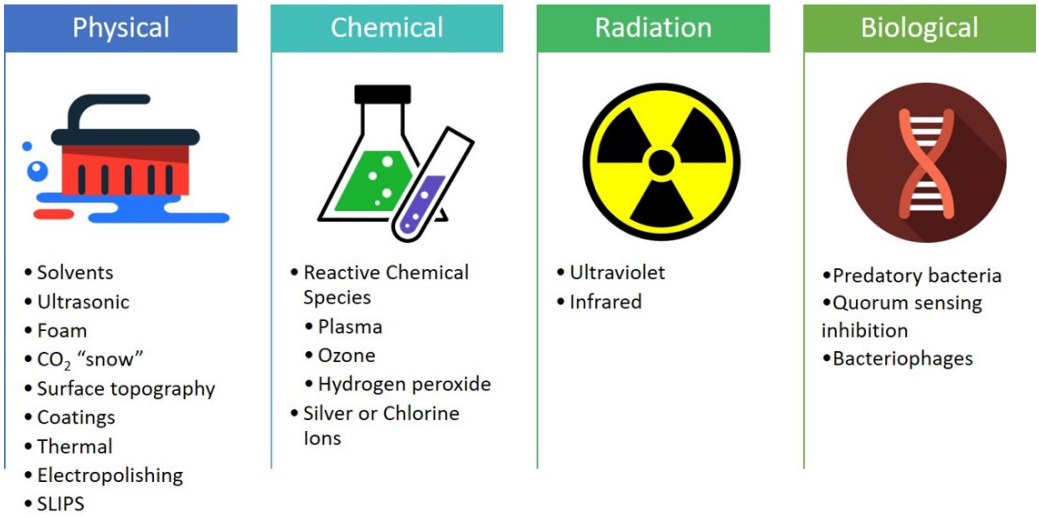

Figure 4. Main methods of implementation under which proposed biofilm control applications fall.

A. Physical and Chemical Methods

Physical methods of biofilm destruction and removal are straightforward in that ideally, a way to physically scrub the biofilm both away from the material and out of the closed loop system would allow the least amount of tampering with the actual water source, therefore lowering the need for reinforcing applications of biocide. However, opening areas in which biofilms have been known to form can not only be difficult to reach and scrub, but can expose the

system to the microbial environment of the recycled air and allow other strains of bacteria to settle in before being closed back up. Without opening the system, flushing would be the most practical cleaning method but considering how some biofilms are known to attach more strongly in high shear forces[43], this could potentially do more damage than good if not done properly.

In extension of this, flushing hardware with solvents could dissolve the biofilm but removal would once again be difficult due to the inability to fully extract the biofilm remnants, unless a supplemental application of sonication could be used,[54] or a form of other physical means of removal is applied. CO_2 "snow" jets, according to Pugel et al.[54] could also be used to blast micron-sized contaminants away as a jet in heat or chemically sensitive areas. However scaling up is difficult and the jet does not get rid of or damage spore formations, much like many solvents. This could limit their use to only inhibition instead of total biofilm removal if spores are present, unless more sporicidal chemicals are added. Also, if required, the sporicidal solutions could affect water quality and toxicity and would require further investigation depending on the combinations and amounts used. Certain hardware could also be treated thermally, though considerations of heat tolerance and spore survival could make the process inefficient. Steam and dry heat have been used in the medical field effectively,[55] but the practicality and time requirement of such a method of sterilization could make it difficult in application to a system in space. In cases where spores are found to be an issue, it might help to study what drives microbes to create spores to help avoid using more powerful antimicrobials. To our knowledge, the presence of spores has not been of major concern and therefore not as studied.

When considering the materials onto which biofilms are more prone to attach, changing the surface of interest can be an option. Considered a passive and sometimes hybrid of physical and chemical approaches, this could include creation of surfaces which discourage bacterial adhesion (negating surface interactions) without further influence or could rely on environmental changes (pH or heat). Chang and colleagues[56] found that biofilm formation could be discouraged by limiting oxygen availability and changing surface properties including shape and hydrophobicity utilizing a silicone-elastomer blended material which could be prepared in several different shapes. Renner and Weibel[57] did a review in which a polymer coating of poly(ethylene oxide) was mentioned as being found to inhibit protein adsorption and repel adhesion of bacteria sterically, leading to reversible attachment. A review by Hasan and Chatterjee[58] also mentioned some of the surface treatments which are being developed with biomimicry of shark skin, lotus or rice leaves, and insect wings. Such biological surfaces have been found to rely on superhydrophobicity to repel not only water but bacteria attempting to attach. Geyer et al.[59] utilized a silicone nanofilament coating on tubes, which was polymerized on the surface to make the methyl groups present there orient themselves in such a way as to lower surface energy, rendering the coating superhydrophobic and successfully deterring growth of *E.coli.* Creation of surfaces treatments overall could lead to less upkeep, but further study into their scalability, maintenance, and propensity for surface defects (which bacteria could then latch on to) are also required if they are to be used as long term solutions in biofilm control.

Other findings by Hasan et al.[60] about dragonfly wings have revealed arrays of pillars which are successful at not only being superhydrophobic, but at leading to rupture of bacteria attempting to attach. Such mechanisms would require a method to get rid of the ruptured cell bodies to not encourage growth in areas where they may cover the pillars and therefore negate the bactericidal effect of the topography. The review by Renner and Weibel[57] also discussed lysing of microbes through attachment of bactericidal molecules covalently to substrate surfaces made from quaternary ammonium groups (N,N′-disubstituted poly(ethyleneimine) or N-substitute polyvinylpyridine polymers). The positive charge of the chains would be attracted to bacterial membranes and cell walls, with flexibility which would allow them enough freedom to attach, ultimately causing cell lysis. Such a method did not seem to discriminate between cell types and therefore would require some form of optimization to ensure attachment to the desired cells only. If there was any potential of the attached groups to come off and reach the end user of the water system somehow, knowledge of potential effects (if any) would need to be studied.

Other studies are looking instead to keep the bacteria from ever attaching to surfaces. Epstein et al.[61] looked at creating a lubrication layer between the substrate surface and bacteria inspired by the *Nepenthes* pitcher plant. Called a slippery liquid-infused porous surface (SLIPS). The material uses chemical affinity between the lubricating fluid and substrate (which should be higher than that of the fluid being run through the system and the substrate), immiscibility between lubricant and system fluid, and increased surface area of the substrate to keep the lubricant both attached to the substrate only and repellant of anything in the system fluid. In another study, Cheng et al.[62] utilized long-chain zwitterionic surfaces created through atom transfer radical polymerization to discourage bacterial attachment. Zwitterionic materials have negative and positively charged groups present but an overall neutral charge, though they are known to be hydrophilic in nature.[63] Their hydrophilicity thus helps create a layer of hydration which then acts as a barrier against bacterial adhesion. Another coating chemical could be phosphorylcholine, which is highly hydrophilic, leading to higher absorption of water and creating a water barrier that would block bacterial adhesion.[64]

Electropolishing is another surface treatment that does not destroy bacteria directly. Used to clean materials such as pipes, valves, and things made of stainless steel, electropolishing relies on the removal of a surface layer of the desired material. This is accomplished by creating a sacrificial layer by running a current between the material (acting as an anode) and an inserted cathode.[65] The material therefore goes through oxidation that dissolves the surface into a surrounding electrolyte solution before being pulled into a reduction process at the cathode. Removal of the surface layer then could remove the biofilm as it creates a smooth surface.[66] With all of these approaches, biofilms cannot be formed permanently through attachment to the water system surfaces, but the microbes are not harmed in any way. How the bacteria would adapt to such changes would hence require further study.

Chemical biocides include compounds such as silver, hydrogen peroxide, chlorine, and ozone.[6] Of these, silver is considered one of the methods being most studied for state-of-the-art water treatment, both to be adaptable between the ISS Russian and American segments as well as because of the ability to apply silver through innovative and practical methods. Still, how to keep it from depleting quickly remains a challenge. And even though it has been shown to be a good chemical disinfectant, its antibacterial mechanisms are still being studied for optimization due to the ability of microbes to increase their resistance.[67] Approaches with silver as nanoparticles can help optimize biocides without reaching high enough concentrations to be toxic and yet be a long term self-sustained solution that is easy to replenish. NASA is developing a passive biocide delivery system that consists of a silver nanoparticle composite foam, in which silver oxide and silver chloride nanoparticles are incorporated into a polyurethane foam matrix. This would allow for dispersal of the nanoparticles while still being protected enough from the flow of water so that the whole nanoparticle load of silver ions is not dispersed at once. Assuring the rate of silver ion delivery would keep the toxicity levels low while still permitting the flow of water through the matrix. It would also decrease potential interactions of silver with the surrounding material (pipes, tubing, etc.) which could lower the efficiency of the biocide.

Hydrogen peroxide works by free-radical oxidation and is valuable in that its byproducts after bactericidal activity are simply water and oxygen. Currently, production of hydrogen peroxide is undergoing optimization for higher scale operations due to the energy intensive steps required for the process as well as lack of stability of the produced compound.[68] Bromine and chlorine are also known to work via oxidation but can be sensitive to pH changes and cause corrosion of materials.[69] While chlorine has been applied in municipal water systems, application in space is less likely due to the safety concerns created by the chemical transportation and storage on spacecraft. Ozone is a reactive chemical species reliant on one of its oxygen moieties for bactericidal characteristics. Generation of ozone is achieved by exposing oxygen to high energy sources such as high voltage, UV radiation, or electric currents and utilizes oxidation reactivity by decomposing into free radicals which are effective against a variety of bio-material (spores, biofilm components, viruses).[70] The application of ozone is of interest because like hydrogen peroxide, ozone creates oxygen and water as byproducts. Overall, though there are a variety of different chemical and physical methods to destroy and remove biofilms, it seems that optimization of these applications will require both synergistic approaches and the use of nanotechnology to reduce bacterial adaptation and resistance without leading to increase in potential toxicity for the end user of the water recovery system.

B. Radiation Methods

Radiation methods have not been directly used in the potable water system but have been considered. Infrared radiation has not seen much application specifically for water systems due to the focus being on medical application development.[71] However, in a study by Yin et al.[72] a plasmonic molybdenum oxide sheet was used to absorb near infrared light which would then be converted to heat (inducing photothermal lysis) and creating reactive species. When in conjunction with silver nanocubes, the bactericidal effect was not only raised simply by the presence of the silver but also by the increased silver ion release with near infrared light application. Though only tested with two types of microbial species (*E. coli* and *S. aureus*), the potential of the proposed material is a great example of how infrared radiation can be exploited as a photocatalyst with coatings.

Plasma is a gas that can be partially or fully ionized and can generate hydrogen peroxide, ozone, free radicals, ions, and electrons. Electrical discharge plasma specifically has been applied for terrestrial water treatment because of its ability to generate very reactive species, without further chemical assistance, and because of how tunable the parameters are for controlled release.[73] Still, the energy efficiency is known to be a problem in application due to the complex interplay of water properties (such as pH, conductivity, or present salts), source used for excitation, and the gas which is being applied to the process.[54,73]

C. Biological Methods

Biological methods of biofilm mitigation are one of the more novel approaches in development. For instance, the application of predatory bacteria housed within bioreactors has been proposed recently for the Urine Processor

Assembly (UPA) waste water treatment.[74] Using the predatory bacteria proposed in the study as an example, de Vries[74] offered two mechanisms of potential attack of the undesired biofilm microbes, including entrance into the bacteria with eventual lysis (*Bdellovibrio bacteriovorus*), versus attachment to prey bacteria and leeching of nutrients (*Micavibrio aeruginosavorus*). Control of predatory bacteria does come with the question of whether their mutation can lead to resistance to antimicrobials, which could indicate a need for more control than other biocidal systems if studies show any indication of higher possibility of this to occur. Also, how they would interact with the rest of the water recovery system (materials, water flow, tanks) would require support studies to show that they are only attacking the desired prey.

The quorum sensing abilities of bacteria, which are inter or intracellular communications via signaling molecules that help mitigate metabolism, are also an emerging area of study.[75,76] Vega et al.[76] did a study on a heat exchanger (SPCU HX) from the ISS extravehicular mobility unit (EMU) and found that even though the coolant fluid had a high microbial load, no biofilm formation was present. Based on their results, they suggest that the materials of the heat exchanger itself could have been inhibiting biofilm formation through influence on bacterial quorum sensing mechanisms. More research is currently being done in hopes of better biofilm control of waste water membranes and membrane bioreactors terrestrially with natural and synthetic quorum ability inhibitors,[77] but application on materials of other sections of water recovery processes remains undeveloped. How these methods will be affected by the lack of gravity and related environmental effects in a system of water also remains to be elucidated, though the studies from the previous section of our work would be most likely applicable directly through this type of inhibition.

The final method of biological mitigation of biofilms, that is seeing more growth is the utilization of bacteriophages. To our knowledge, no work in this area of study has been proposed for space research. Terrestrially, though they have seen development for application in water system treatments, their foremost progress is in medical applications.[78,79] Bacteriophages are viruses known for their ability to infect bacteria specifically.[80] With bacteria able to adapt to their stressor more readily, the use of bacteriophages offers a form of bacterial mitigation that can be specific to certain bacteria, with the ability to replicate in the environment only when their bacterial food source is available. Though there still is a possibility that bacteria can develop resistance, use of a "cocktail" of different phages, or cycling with several types, could help overcome the issue. Biofilms can also be a physical barrier that could pose a challenge, but utilizing phages which can bypass the diffusion barrier of a biofilm, through polyvalency and low adsorption rates, could help. The biggest issues with use of bacteriophages seem to lie mainly with the determination of which strains of bacteria are present in the problematic community and the state of the water environment. Temperature, salinity, and pH could all affect bacteriophage characteristics before they even come in contact with their intended target.[80]

Taking into account the studies done on microbial growth in the previous section, an unexplored avenue could also include not aiming to *attack* biofilm formation, but to *guide* it to occur in certain areas which are easier to reach and disinfect, while treating bacteria-friendly surfaces to be less likely to welcome growth. This could require a more time-consuming approach, in which in-depth knowledge for tailoring of the environment of bacterial communities (such as nutrition and surface properties), would be applicable. If the bacteria could be grown on a substrate which could be easily removable, they could be allowed to grow until they reach a threshold prior to beginning the process of encouraged planktonic microbe release.

V. Summary

Biofilm formation in space has been a persistent problem onboard spacecraft and has led to system hardware impairment and medical issues. Although bacterial growth in space has been studied, understanding of behavioral changes under microgravity conditions remains unclear. Studies seem to suggest that determination of motility can be one of the initial factors which can help predict whether the environment will influence microbial behavior. Further research also appears to indicate that biofilm structure determination can be driven by the availability of nutrients with motility prompting certain structure formations. Microbial genetic variations occurring in space further suggest reactions to their environment similar to terrestrial conditions based on nutrient availability. Clarification of microbial behavior could help find a long term and stable method of biofilm inhibition, with state-of-the-art methods appearing to be taking on more synergistic mechanisms to address microbial adaptability. Making sure new approaches are self-sustainable, material friendly, and overall safe for crew consumption will be crucial considerations in order to successfully achieve mitigation of microbial growth on future missions.

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